RESEARCH ARTICLE DEPRESSION

A phase 2 trial of inhaled nitrous oxide for treatment-resistant major depression

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Laughing gas improves depression

About one-third of individuals suffering from depression are at risk for treatment resistance. Whereas inhaled 50% nitrous oxide has early antidepressant effects on individuals with treatment-resistant major depression (TRMD), adverse effects can occur at this concentration. In this phase 2 clinical trial, Nagele *et al*. studied the effects of a single 1-hour treatment with 25% nitrous oxide on depression symptoms in those with TRMD, finding that this lower concentration had comparable efficacy to 50% nitrous oxide over several weeks but was associated with significantly fewer adverse effects. These results highlight that lower concentrations of nitrous oxide may be a useful treatment for TRMD.

Abstract

Nitrous oxide at 50% inhaled concentration has been shown to improve depressive symptoms in patients with treatment-resistant major depression (TRMD). Whether a lower concentration of 25% nitrous oxide provides similar efficacy and persistence of antidepressant effects while reducing the risk of adverse side effects is unknown. In this phase 2 clinical trial (NCT03283670), 24 patients with severe TRMD were randomly assigned in a crossover fashion to three treatments consisting of a single 1-hour inhalation with (i) 50% nitrous oxide, (ii) 25% nitrous oxide, or (iii) placebo (air/oxygen). The primary outcome was the change on the Hamilton Depression Rating Scale (HDRS-21). Whereas nitrous oxide significantly improved depressive symptoms versus placebo ($P = 0.01$), there was no difference between 25 and 50% nitrous oxide ($P = 0.58$). The estimated differences between 25% and placebo were −0.75 points on the HDRS-21 at 2 hours ($P = 0.73$), −1.41 points at 24

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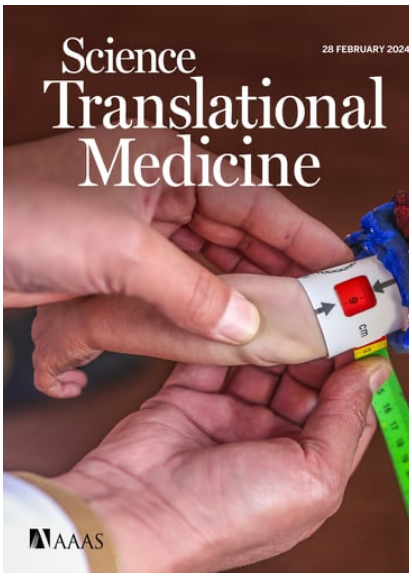

hours ($P = 0.52$), −4.35 points at week 1 ($P = 0.05$), and −5.19 points at week 2 ($P = 0.02$), and the estimated differences between 50% and placebo were −0.87 points at 2 hours ($P = 0.69$), −1.93 points at 24 hours ($P = 0.37$), −2.44 points at week 1 ($P = 0.25$), and −7.00 points at week 2 ($P = 0.001$). Adverse events declined substantially with dose ($P < 0.001$). These results suggest that 25% nitrous oxide has comparable efficacy to 50% nitrous oxide in improving TRMD but with a markedly lower rate of adverse effects.

INTRODUCTION

Treatment-resistant major depression (TRMD) is a severe form of major depressive disorder (MDD) in which patients fail to respond to multiple standard antidepressant treatments (*1*, *2*). The lifetime prevalence of MDD is estimated to be about 10 to 20%, of which at least one-third of patients are estimated to be at risk for TRMD (*3*–*5*). For the United States alone, this equates to about 17 million adults with TRMD (*6*).

A proof-of-principle study demonstrated that a 1-hour inhalation of 50% nitrous oxide ("laughing gas") has rapid antidepressant effects in patients with TRMD (*7*). The study had two important limitations. First, it did not formally test whether antidepressant effects lasted beyond 24 hours. Second, it used a high concentration of nitrous oxide (50%), for which risk of nausea and other unwanted side effects may limit its clinical use. Moreover, evidence from the antidepressant use of ketamine (*8*), a drug with a similar proposed mechanism of action (*N*-methyl-D-aspartate receptor antagonism) (*9*–*11*), suggests that a lower subanesthetic dose may be equally efficacious while potentially conferring a lower risk of side effects (*12*–*14*).

In this current trial, we sought to determine whether a lower concentration of nitrous oxide (25%) had comparable antidepressant efficacy in TRMD as 50% nitrous oxide. Additional goals of this investigation were to determine whether inhalation of 25% nitrous oxide would be associated with fewer side effects and whether the antidepressant effects of nitrous oxide would extend beyond 24 hours after a single inhalation treatment with a follow-up period of at least 14 days.

RESULTS

Recruitment and characteristics of enrolled patients

After excluding 6 patients for screen failure, 28 patients were enrolled between November 2016 and October 2019 (CONSORT diagram; fig. S1). Twenty patients completed all three inhalation sessions (placebo, 25% nitrous oxide, and 50% nitrous oxide); one patient withdrew after two sessions. Three patients withdrew after one session, and four patients withdrew after screening but before any treatment (table S1 lists adverse events and reasons for withdrawal). Results are reported from the 20 patients who completed all three inhalation sessions (60 total inhalation sessions) and the 4

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who completed all three inhalation sessions (66 total inhalation sessions) and the 1 patients who received at least one treatment. One patient inadvertently received two sessions with 50% nitrous oxide and no placebo, resulting in a final number of 22 treatments with placebo, 20 treatments with 25% nitrous oxide, and 23 treatments with 50% nitrous oxide.

Study patients had sustained and refractory depressive illness with an average of 17.5 lifetime years of MDD and having failed a median of 4.5 [interquartile range (IQR), 3 to 10] adequate-dose/duration antidepressant drug treatments (Table 1). The median HDRS-21 (Hamilton Depression Rating Scale, 21 items) score at enrollment was 20.5, and the median MADRS (Montgomery-Asberg Depression Rating Scale) score was 30, indicative of severe TRMD.

Age (years)	**39 [26–68]**
Female sex [no. (%)]	17 (71%)

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Table 1 Baseline characteristics of participants [*n* (sample number) = 24].
Numbers are listed as medians and interquartile range (IQR) or counts and percentages. ECT, electro-convulsive therapy. rTMS, repetitive transcranial magnetic stimulation. SSRI, selective serotonin reuptake inhibitor; SNRI, serotonin-norepinephrine reuptake inhibitor.

Primary and secondary study outcomes

In the intention-to-treat analysis (n = 24, 20 completers and 4 partial completers), the overall effect of nitrous oxide (both groups) compared to placebo on the primary outcome (HDRS-21) over the course of 2 weeks of observation was significant (P = 0.01), but there was no significant difference between 25 and 50% nitrous oxide (P = 0.58). The estimated differences between 25% and placebo were −0.75 points (HDRS-21) at 2 hours (P = 0.73, d = 0.16), −1.41 points at 24 hours (P = 0.52, d = 0.21), −4.35 points at week 1 (P = 0.05, d = 0.38), and −5.19 points at week 2 (P = 0.02, d = 0.62). The estimated differences between 50% and placebo were −0.87 points at 2 hours (P = 0.69, d = 0.29), −1.93 points at 24 hours (P = 0.37, d = 0.32), −2.44 points at week 1 (P = 0.25, d = 0.35), and −7.00 points at week 2 (P = 0.001, d = 0.85) (Fig. 1). The estimated differences between 50 and 25% were 0.11 points at 2 hours (P = 0.96), 0.42 points at 24 hours (P = 0.85), 1.91 points at week 1 (P = 0.37), and −1.67 points at week 2 (P = 0.44). The estimated differences between placebo and the combined 25 and 50% groups were −0.81 points at 2 hours (P = 0.66), −1.67 points at 24 hours (P = 0.37), −3.35 points at week 1 (P = 0.07), and −6.13 points at week 2 (P = 0.001). Relative to placebo, the effects of the active treatment groups linearly increased over time for the 25% group (−1.38 per measurement occasion; P = 0.007) and for the 50% group (−1.55 per measurement occasion; P = 0.002). A significant dose-response relation was found at week 2 (a −3.51 decrease per 25% increase in dose; P = 0.001) but not at earlier measurement times. To study carryover effects, we performed an analysis to determine whether order of receipt of the 50% dose was related to the 2-week HDRS-21 score. No significant effect of trial order was found (P = 0.22).

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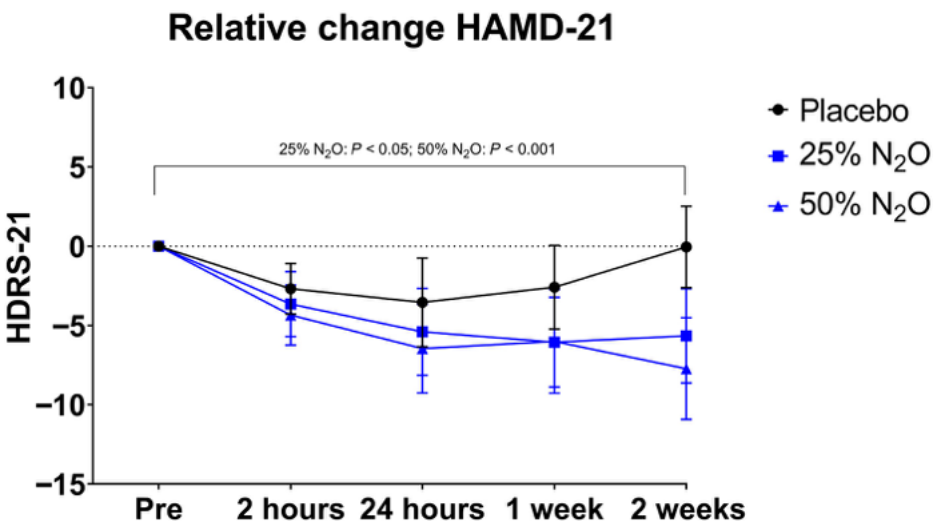

Fig. 1 Relative change in depressive symptoms between 50% nitrous oxide, 25% nitrous oxide, and placebo on the Hamilton Depression Rating Scale.
In the intention-to-treat analysis ($n = 24$) using a mixed-effects linear regression model, the overall effect of nitrous oxide (both groups) compared to placebo was significant ($P = 0.01$), but there was no significant difference between 25 and 50% nitrous oxide ($P = 0.58$). Means ± 95% CI.

The MADRS data were similar in efficacy to the HDRS-21 results regarding 50% nitrous oxide but not for 25% nitrous oxide (not significant). Results on the QIDS (Quick Inventory of Depressive Symptomatology) scale were, except for 50% nitrous oxide at 2 weeks, not statistically significant. Results on the POMS (Profile of Mood States) scale showed a stronger response at 50% nitrous oxide (which is significant at 2 weeks) but not for 25% nitrous oxide. (fig. S2).

Over the entire course of treatment (including only patients who completed the study; $n = 20$), patients experienced a clinically significant improvement in depressive symptoms from a median baseline HDRS-21 score of 20.5 (IQR, 19.0 to 25.5) to 8.5 (IQR, 2.0 to 16.0) at study completion, corresponding to a median change of −11.0 points (IQR, −3.3 to −14.0 points; $P < 0.0001$) after the 3-month study period (Fig. 2). At study completion, 11 of 20 patients (55%) had a treatment response (reduction in HDRS-21 points, ⩾50%), 8 of 20 (40%) were in remission (HDRS, ⩽7 points), and 17 of 20 (85%) had an improvement in depressive symptoms by at least one category (for example, from severe to moderate).

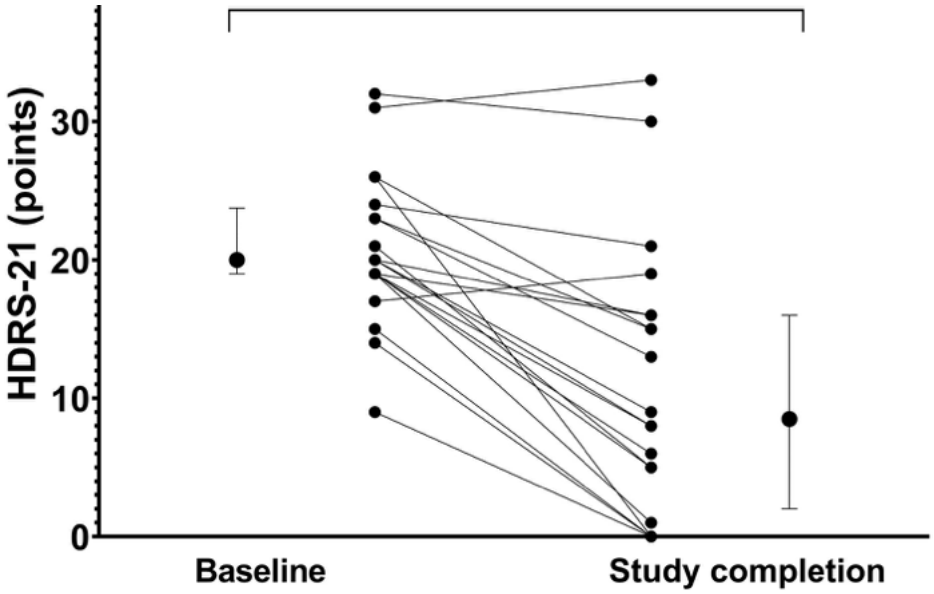

Fig. 2 Comparison of severity of depressive symptoms before and after study completion.
Patients who completed the study (n = 20) experienced a significant improvement in depressive symptoms with a median reduction of the Hamilton Depression Rating Scale of 11.0 points upon completion of the study (IQR, −3.3 to −14.0 points; two-sided paired t test, P < 0.0001).

Figure 3 demonstrates the rates of treatment response and remission for each inhalation treatment (in this analysis, we only included treatments where the pretreatment HDRS-21 score was ⩾19). After placebo treatment, one of nine patients had a treatment response (11.1%) and one of nine was in remission (11.1%). After 25% nitrous oxide, three of nine patients had a treatment response [33.3%; relative risk (RR), 2.50; 95% confidence interval (CI), 0.43 to 16.30] and two of nine were in remission (22.2%; RR, 1.82; 95% CI, 0.27 to 12.84). After 50% nitrous oxide, 5 of 12 patients had a treatment response (41.7%; RR, 2.94; 95% CI, 0.57 to 18.02) and 5 of 12 were in remission (41.7%; RR, 2.94; 95% CI, 0.57 to 18.02).

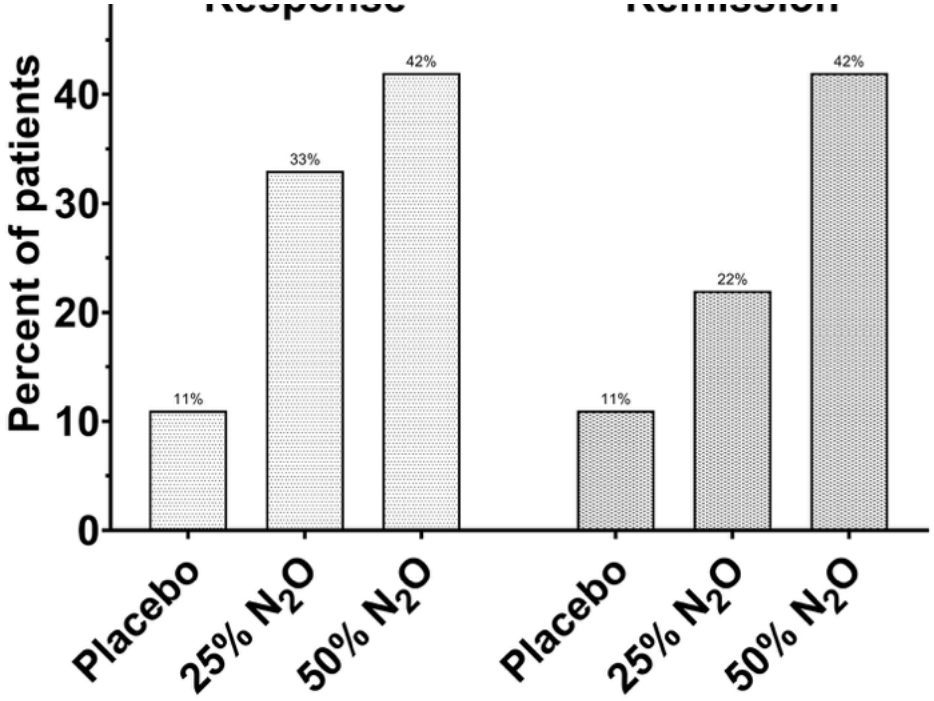

Fig. 3 Proportion of patients who experienced response or remission after treatment with 50% nitrous oxide, 25% nitrous oxide, and placebo.
In this analysis, we only included treatments where the pretreatment HDRS-21 score was ≥19. Differences between categorical outcomes (response and remission) were tested by Fisher's exact test; RR and 95% CIs were calculated using the Koopman asymptotic score. After placebo treatment, one of nine patients had a treatment response (11.1%) and one of nine was in remission (11.1%). After 25% nitrous oxide, three of nine patients had a treatment response (33.3%; RR, 2.50; 95% CI, 0.43 to 16.30) and two of nine were in remission (22.2%; RR, 1.82; 95% CI, 0.27 to 12.84). After 50% nitrous oxide, 5 of 12 patients had a treatment response (41.7%; RR, 2.94; 95% CI, 0.57 to 18.02) and 5 of 12 were in remission (41.7%; RR, 2.94; 95% CI, 0.57 to 18.02).

We assessed the quality of blinding by including a questionnaire after each inhalation session in which patients were asked whether they were receiving active treatment or placebo. In 50 of 60 sessions (83%), patients correctly guessed their treatment group and 8 of 60 (13%) guessed incorrectly, and in two instances, patients did not know. Review of the records demonstrated that four patients either added (n = 2) or increased (n = 2) antidepressant dosages during the course of the study, four patients had confirmed antidepressant medication decreases, and two patients had confirmed antidepressant medication discontinuation.

Safety and adverse effects of inhaled nitrous oxide

We observed a statistically significant difference in adverse events between treatments: 47 adverse events after inhalation with 50% nitrous oxide, 11 adverse events after inhalation with 25% nitrous oxide, and 6 adverse events after placebo inhalation (P < 0.0001). None of the adverse events were serious, and nearly all occurred either during or immediately after the treatment session and resolved within several hours (Table 2).

Adverse event	50% N_2O (n = 23)	25% N_2O (n = 20)	Placebo (n = 22)
I. During or immediately after inhalation session			

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Table 2 Adverse events in participants.

DISCUSSION

In this randomized controlled phase 2 crossover trial, we made several key observations. First, the trial extends the original findings in that a single 1-hour inhalation of nitrous oxide, at either 50 or 25%, provides rapid antidepressant efficacy in patients with severe TRMD (*7*). Second, the antidepressant effects increased in magnitude over time, lasting up to 4 weeks in some patients. Third, the trial demonstrated a high rate of response, remission, and symptom improvement in a population of severely TRMD depressed patients: Three months from study initiation at completion, 85% of patients had improved, 55% had a treatment response, and 40% were in remission. Fourth, 25 or 50% nitrous oxide showed equivalent antidepressant efficacy; however, 25% nitrous oxide demonstrated a markedly lower rate of adverse side effects. Fifth, although the two dosages demonstrated roughly equivalent antidepressant efficacy, there was evidence of a dose-response relationship at the 2-week follow-up. Sixth, individual time trends showed considerable interindividual variability; however, incorporating this variability in our statistical models did not mute the significance of the treatment-related effects.

Although we observed antidepressant effects in most patients after nitrous oxide inhalation, the response was not uniform. Some patients had minimal or no improvement after nitrous oxide and placebo and should be considered nonresponders. Furthermore, some patients had a strong placebo response, which, in some instances, mirrored the response to nitrous oxide. We observed that despite a 4-week interval between inhalation sessions, some patients showed sustained improvement of their depressive symptoms and did not return to their pretreatment baseline level of depression severity. Whereas inhalation with 25% nitrous oxide was statistically similar to 50%, on average, there was a tendency toward greater improvement in depressive symptoms in the 50% nitrous oxide group at 2 weeks after treatment. In addition, the antidepressant response to nitrous oxide on the self-reported POMS scale, which measures immediate mood effects, supports that patients reported stronger efficacy of 50% as compared to 25% nitrous oxide. The majority of patients saw a marked improvement of their depressive symptoms throughout completion of the study where each received two nitrous oxide and one placebo treatment over the course of 3 months. Whereas a Hawthorne effect (being in a clinical study) and placebo effects may explain some of the observed improvement, an alternative explanation may also be that a series of two nitrous oxide treatments may have additive and sustained efficacy compared to a single nitrous oxide inhalation treatment.

To put the magnitude of these effects into context, (*15*) synthesized the data for 37 adult and geriatric placebo-controlled double-blind randomized trials of fluoxetine and venlafaxine using the HDRS-17 as an outcome. They found an estimated separation of −2.55 HDRS-17 units at 6 weeks between active treatment and placebo control. Given the 24% increased range of the 21-item HDRS relative to the 17-item HDRS, this

Given the 24% increased range of the 21-item HDRS relative to the 17-item HDRS, this is equivalent to a −3.16-unit difference on the HDRS-21 at 6 weeks. Smaller effects were observed at 2 weeks (*15*, *16*). By contrast, we found a difference of −7.00 HDRS-21 units for 50% nitrous oxide and −5.19 units for 25% nitrous oxide at 2 weeks, after a single treatment. This becomes particularly relevant in the context of the trial patient population [this study being severe TRMD and (*15*) being milder depression].

Because this was a phase 2 clinical trial to determine the risk/benefit ratio for 50% versus 25% nitrous oxide for treatment-resistant MDD, one must be cautious in extrapolating the findings of a small trial to a large patient population. However, these trial results suggest that it may be reasonable to start treatment with a lower dose of nitrous oxide (25%) because of its comparable efficacy and lower risk profile but to consider escalating to 50% when a stronger treatment effect is desired. Since the publication of the first trial investigating the potential use of nitrous oxide in the treatment of TRMD, one case report has shown efficacy of a single inhalation of 50% nitrous oxide beyond 1 month (*17*) and early promise in the treatment of posttraumatic stress disorder in U.S. veterans (*18*) and in an experimental model of psychological trauma to simulate posttraumatic stress disorder (*19*).

Whereas the efficacy of 25% nitrous oxide was similar to 50% nitrous oxide, the risk of adverse effects was not. Inhalation of 25% nitrous oxide compared to 50% nitrous oxide was associated with a fourfold decrease in adverse events. Nearly all adverse events occurred during or immediately after the administration of nitrous oxide and were typically limited to a few hours. Adverse events in this study were related to sedation, mild dissociative effects (lightheadedness, paranoia, and feeling high), and nausea and vomiting consistent with prior evidence (*20*, *21*).

There are several limitations to this study. First, the sample size was appropriate for a dose-finding phase 2 trial but small; however, it was not too small to detect significant separation between treated and control patients. Second, the follow-up was limited to 2 weeks and a single treatment. We note, however, that for the entire 3-month treatment regimen, which involved two treatments with nitrous oxide, a median reduction of 11 HDRS-21 points was observed, which is much larger than that typically seen for traditional antidepressant trials (*15*, *16*). Third, despite having a 1-month interval between treatment sessions, some patients had a sustained treatment effect beyond 4 weeks, which resulted in a carryover effect and influenced the study power. Fourth, in more than four of five instances, patients correctly guessed their treatment (nitrous oxide versus placebo), which is higher than expected because of chance. Thus, there is potential for bias in the study results, although, in a similar clinical trial, we did not observe a treatment effect of nitrous oxide for bothersome tinnitus despite 85% of patients correctly guessing the treatment arm (*22*).

Given the calming effects of nitrous oxide, it is extremely difficult to completely blind patients to nitrous oxide versus placebo. Concomitant use of a relaxing agent in the placebo group, for instance, benzodiazepines, to artificially mimic the temporary euphoric/anxiolytic effects of nitrous was considered; however, out of concern that this could differentially affect depressive symptoms, we decided not to use this method (*23*). That said, there was a small placebo effect observed (Fig. 1), which appeared to peak at 24 hours and subside by 2 weeks. In addition, the fact that some patients

changed the dosage or choice of their antidepressant medication may have influenced some study results. Last, the nonsignificant results of 25% nitrous oxide on QIDS and

some study results. Last, the nonsignificant results of 25% nitrous oxide on QIDS and POMS scales raise the concern of functional unblinding because the 50% nitrous oxide inhalation had markedly higher rates of adverse events and would thus have been most easily discriminated from placebo by the participants in the crossover design.

The findings of this study support that a lower concentration of nitrous oxide (25%) has similar efficacy in TRMD, as compared to 50% nitrous oxide, while having a markedly lower risk of adverse events. The antidepressant effects of nitrous oxide may last between 2 and 4 weeks.

MATERIALS AND METHODS

Study design

The study was a single-center, double-blind, randomized placebo-controlled crossover trial. All subjects underwent three 1-hour inhalation sessions in random order, each separated by at least 4 weeks. The sessions included the following: placebo (0% N_2O), 25% N_2O, and 50% N_2O balanced with air/oxygen. Blinding was executed by separating locations and teams for inhalation treatments and psychiatric evaluations. Only the anesthesia team administering the inhalation treatments was aware of study group assignment; all other participants, including patients and raters, were blinded. Likewise, the study setup was identical for all sessions, and gas flow meters were concealed, making inadvertent unblinding unlikely. The study was approved by the Washington University in St. Louis Institutional Review Board, and all patients provided written, informed consent. The trial was registered at clinicaltrials.gov (NCT03283670).

Patient enrollment

Patients were recruited from an existing database of patients with TRMD identified through participation in a secondary referral clinic for TRMD at Washington University Department of Psychiatry, as well as from the "Volunteers for Health" patient pool (individuals with various medical/psychiatric conditions who volunteer to participate in clinical research) within Washington University School of Medicine. Inclusion criteria were as follows: (i) adults 18 to 75 years of age, (ii) current diagnosis of unipolar MDD without psychosis as confirmed by the Mini International Neuropsychiatric Interview, (iii) a score of ⩾19 on the MADRS, (iv) documented lifetime failure to respond to three or more adequate dose/duration antidepressant treatment trials, including one or more antidepressant medication failure(s) in the current depressive episode, and (v) good command of the English language. Exclusion criteria were as follows: (i) meeting criteria for any Diagnostic and Statistical Manual of Mental Disorders diagnosis for schizophrenia, bipolar, schizoaffective, obsessive-compulsive, personality, or panic disorders; (ii) any recent (within past 12 months) history of substance dependence or abuse (except tobacco), determined by reported history and urine drug screen; (iii) ability to become pregnant and not using effective contraception; (iv) contraindication against the use of nitrous oxide; (v) inability to provide informed consent; and (vi) any other factor that in the investigators' judgment may affect patient safety or compliance. Patients were instructed to continue their current standard-of-care MDD treatment and to maintain a stable psychotropic medication dosage or psychotherapy regimen for 4 weeks before initiation of the study and throughout the study. Furthermore, patients were instructed not to modify their antidepressant treatments during the 3-month course of the trial and were discouraged from adding new antidepressants or modifying existing antidepressant dosages.

modifying existing antidepressant dosages.

Study procedures

There were a total of 14 planned study visits for each patient. A screening visit was used to verify eligibility and to collect background information including patient demographics, medical history, vital signs, and a physical exam. A urine sample was collected for a drug screen, and an optional blood draw was collected if the study physician requested tests to confirm patients' safety to participate. A structured clinical interview, the Mini International Neuropsychiatric Interview (*24*), and other psychiatric assessments measuring baseline depression severity were completed by the research team and patients. This screening verified the appropriate primary diagnosis and ruled out excluded diagnoses such as posttraumatic stress disorder, psychotic disorders, severe comorbid personality disorders, and substance use disorders.

After the in-person screen, each of the three treatment sessions consisted of four visits: pre-inhalation mood assessment, inhalation, and post-inhalation follow-up sessions at 22 to 28 hours, 1 week, and 2 weeks. An additional assessment was completed 4 weeks after the final inhalation treatment.

Inhalation sessions

All three inhalation sessions were scheduled for 1 hour. Patients received an admixture of either (i) placebo (air/oxygen), (ii) 25% nitrous oxide in oxygen, or (iii) 50% nitrous oxide in oxygen. Except for the choice of inhalational gas admixture, treatment sessions were otherwise identical. The gas mix was administered via a standard anesthesia face mask through tubing connected to the anesthesia machine or a Food and Drug Administration–approved Porter/Praxair MXR breathing circuit. A small sample connector line was inserted into the face mask, allowing the measurement of inhaled and exhaled gas concentrations. Total gas flow was 2 to 8 liter/min, and nitrous oxide concentrations were gradually titrated upward over the course of the first 5 to 10 min of treatment. Patients were monitored during and after the treatment according to American Society of Anesthesiologists standards, which include continuous three-lead electrocardiography, pulse oximetry, noninvasive blood pressure, and end-tidal CO_2 measurement under the supervision of an attending-level anesthesiologist. After the 1-hour treatment session, patients were monitored in a recovery room for up to 1 hour at which time a study team physician determined whether the patient met criteria for discharge.

Outcomes

Data were collected at (i) baseline (before each inhalation session), (ii) 2 hours, (iii) 24 hours, (iv) 1 week, and (v) 2 weeks after inhalation. Efficacy of the inhalation treatment on mood was measured using the following: HDRS-21 (primary outcome), MADRS, QIDS Self-Report, and the PMS second edition. Other behavioral assessments included an assessment of dissociation, the Clinical Administered Dissociative States Scale (28 items), and an assessment for the emergence of psychosis, the Brief Psychiatric Rating Scale (18 items). These mood and behavioral assessments were performed by a blinded member of the psychiatry study team. In addition, adequacy of the blind was assessed by a blinding questionnaire given after completion of each inhalation session. Treatment safety was assessed by monitoring adverse events related to (i) cardiovascular status, (ii) respiratory function, (iii) central nervous system, and (iv) psychiatric symptoms, particularly the presence of psychotic symptoms.

Statistical analysis

The intention-to-treat analysis was based on mixed-effects linear regression models to accommodate correlation produced by the within-subject crossover design (*25*). The primary analysis included treatment group (placebo, 25%, and 50%), time (baseline, 2 hours, 24 hours, 1 week, and 2 weeks after inhalation), period, and the treatment-by-time interaction as categorical variables (random intercept model). The period effect adjusted for the cumulative effect of treatment (in random orders) over the course of the study. The treatment-by-time interaction tested the null hypothesis of no difference between the treatment groups in the severity of depressive symptoms (HDRS-21) over time using a likelihood ratio chi-square statistic. The same analysis was performed to directly compare the 25% versus 50% treatment groups to each other and the combined 25 and 50% dosage groups to placebo. These models also provided point in time between group comparisons using Wald tests. A comparison of linear time trends between each of the active treatment groups and placebo was also performed. Last, we also tested for a linear dose-response relation. Differences between categorical outcomes (response and remission) were tested by Fisher's exact test; RR and 95% CIs were calculated using the Koopman asymptotic score. Differences in the count of adverse events were compared by Kruskal-Wallis test. All reported *P* values are two sided, and a *P* value of <0.05 was considered statistically significant. All analyses were conducted using SuperMix (Scientific Software International).

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Supplementary Material

Summary

Figs. S1 and S2

Table S1

Resources

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